AWARD NUMBER: W81XWH-10-1-0872

TITLE: "Inhibition of Orthopaedic Implant Infections by Immunomodulatory Effects of Host Defense Peptides"

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REPORT DATE: December 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2014	Final	15 Sept 2010 - 14 Sept 2014

4. TITLE AND SUBTITLE
"Inhibition of Orthopaedic Implant Infections by Immunomodulatory Effects of Host Defense Peptides"

5a. CONTRACT NUMBER
W81XWH-10-1-0872

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRMC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We first developed a murine model of orthopaedic implant infection that will lead to future studies utilizing transgenic and knock out mice to understand the mechanisms that contribute to the effects of bacteria on implant osseointegration. Both *S. aureus* and *A. baumanii* were found to impair implant osseointegration by inducing local osteolysis. This reconciles confusion in the literature regarding the effects of *A. baumanii*. The effects of *A. baumanii* on osseointegration were found to require quorum sensing. This will lead to future studies to determine whether quorum sensing antagonists that are being developed by other investigators will block the effects of *A. baumanii* on osseointegration. It was also found that the IDR peptide reduces the effects of *S. aureus* on implant osseointegration. This will lead to future studies to optimize the use of IDR peptides in our murine model of implant infection; in larger, more clinically relevant, animal models; and ultimately in human trials.

15. SUBJECT TERMS
Orthopaedic infections, Host Defense Peptides, Murine model, *Staphylococcus aureus*, *Acinetobacter baumannii*

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. **INTRODUCTION:**

Host defense peptides represent a promising new approach to inhibit infection. The anti-infective actions of these peptides are primarily due to their immunomodulatory effects. Since they directly target the mammalian immune system rather than the bacteria themselves, host defense peptides are also less likely to induce bacterial resistance than are traditional antibiotics. The purpose of this project is to assess whether host defense peptides are a promising strategy for treating infected orthopaedic implants. The scope of the project is to measure effects of host defense peptides on macrophages *in vitro* and on implants infected with *Staph. aureus* or *Acinetobacter baumannii* in our murine model of implant osseointegration.

2. **KEYWORDS:**

Orthopaedic infections
Host Defense Peptides
Murine model
Staphylococcus aureus
Acinetobacter baumannii

3. **OVERALL PROJECT SUMMARY:** The following project summary is based on the revised SOW that was approved in conjunction with the EWOF:

Aim 1: Test the hypothesis that soluble host defense peptides reduce infection of orthopaedic implants.

Task 1: Establish a murine model of implant infection
Subtask 1A: Prepare animal use protocol for murine experiments for Tasks 1, 2 & 5: **Completed**

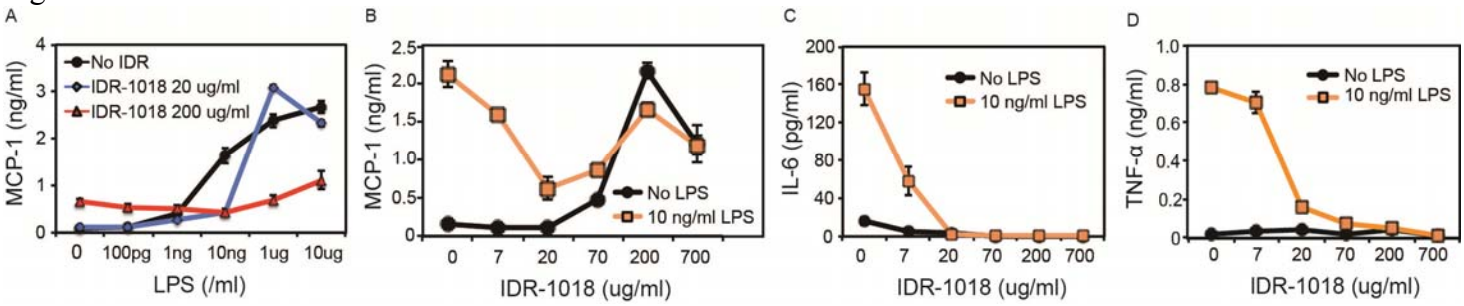
Subtask 1B: Perform and analyze *S. aureus* experiments:
Completed: *S. aureus* impairs osseointegration, see Appended Poster #1 and Appended Publication

Subtask 1C: Perform and analyze *A. baumannii* experiments:
Completed: *A. baumannii* impairs osseointegration, see Appended Posters #3 and #4

Alternative Subtask 1D: Alternative bacteria dosages: **Not Needed**

Task 2: Determine whether soluble host defense peptides reduce implant infection
Subtask 2A: Obtain IDR & inactive analogue and purify, if detectable LPS: **Completed**
Subtask 2B: Confirm in vitro activity of peptides:
Completed: As expected based on the literature, IDR peptide inhibits production of pro-inflammatory cytokines (Fig 1C-D) and induces production of MCP-1 in the absence of pro-inflammatory stimuli (Fig 1A-B). However, we also made the novel finding that the IDR peptide reduces production of MCP-1 induced by other inflammatory stimuli, such as lipopolysaccharide (LPS) (Fig 1A-B). Thus, in the context of a bacterial infection, the effect of IDR on MCP-1 production likely depends on the relative concentrations of IDR peptide and other inflammatory stimuli. see Appended Publication

Fig 1:



Subtask 2C: Perform & analyze in vivo IDR experiments with *S. aureus*:

Completed: IDR peptide reduced bacterial burden (Fig 2) and thereby increased osseointegration (Fig 3). , see Appended Poster #2 and Appended Publication

Fig 2:

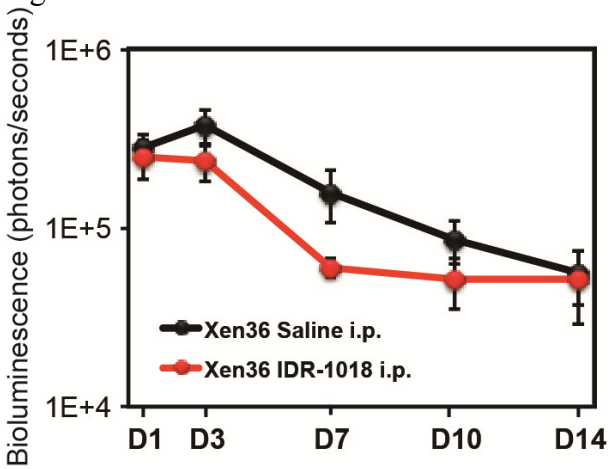
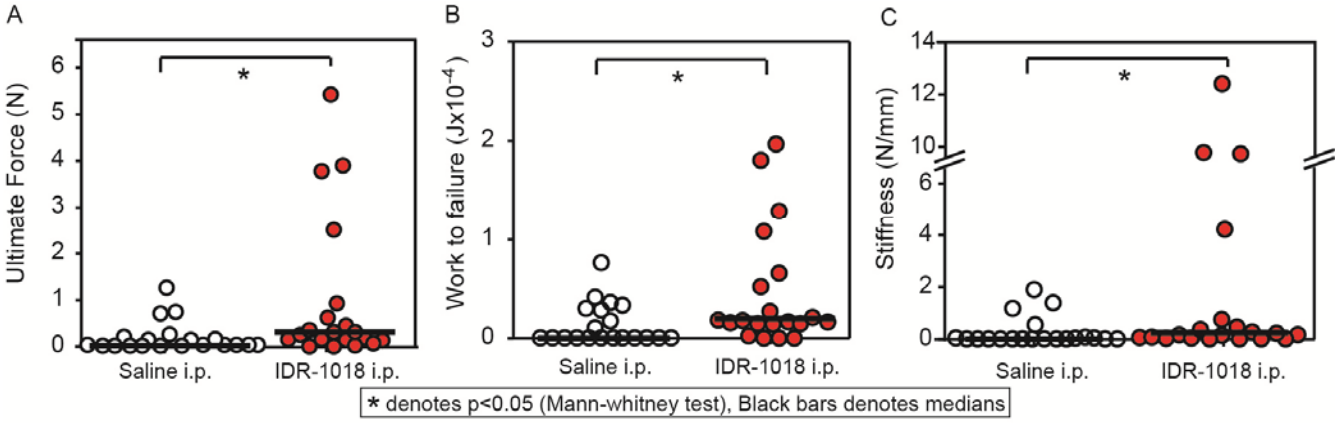


Fig 3:



Subtask 2D: Perform & analyze in vivo IDR experiments with *A. baumannii*: **In progress**

Alternative Subtask 2E: Alternative IDR dosing regimens: **Not needed**

Alternative Subtask 2F: Alternative host defense peptides: **Not needed**

Subtask 2G: Prepare & revise report(s)/manuscript(s) with results from Tasks 1 & 2: **In progress**

Aim 2: Test the hypothesis that host defense peptides bound to orthopaedic implant surfaces reduce infection.

Task 3: Determine which binding motifs/domains bind to implant surfaces

Subtask 3A: Obtain tagged peptides with:

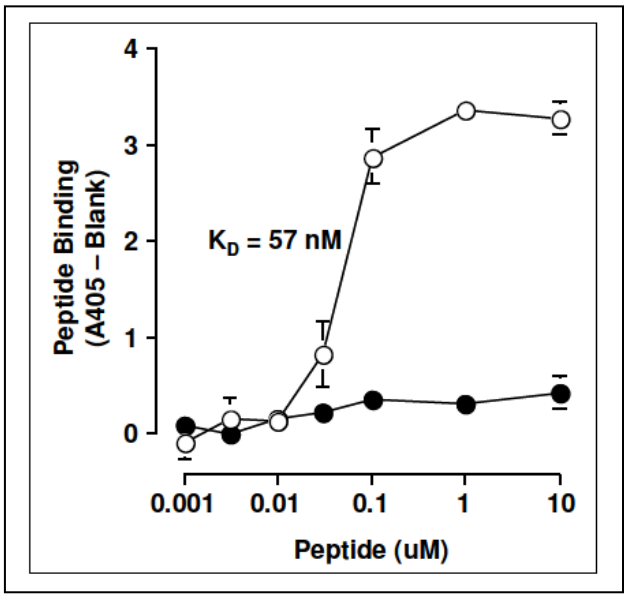
titanium binding motifs: **Completed**

heparin binding domains: **Not needed**

Subtask 3B: Measure binding of peptides with titanium binding motifs to surfaces:

Completed: Biotinylated-3xHKH peptide bound with high affinity to titanium alloy particles (open circles in Fig 4). In contrast, a control peptide showed no detectable binding (filled circles in Fig 4).

Fig 4:



Subtask 3C: Establish protocol for functionalizing surfaces with heparin: **Not needed**

Subtask 3D: Measure binding of peptides with heparin binding domains to functionalized surfaces: **Not needed**

Alternative Subtask 3E: Alternative binding strategies: **Not needed**

Task 4: Determine whether host defense peptides bound to implant surfaces retain immunomodulatory activity.

Subtask 4A: Prepare & revise protocol to obtain human mononuclear cells: **Completed**

Subtask 4B: Establish protocols to measure expression by monocytes of chemokines/cytokines: **Completed**

Subtask 4C: Obtain fusion peptides with IDR-1 & binding motifs/domains and purify, if detectable LPS: **Completed**

Subtask 4D: Measure effects of bound IDR-1 on expression of chemokines and cytokines: **In progress**

Alternative Subtask 4E: Alternative host defense peptides: **Not needed**

Subtask 4F: Prepare & revise report(s)/manuscript(s) with results from Tasks 3-4: **Partially complete,** see Appended Publication (a second manuscript is in preparation)

Task 5: Determine whether bound host defense peptides reduce implant infection.
Subtask 5A: Perform & analyze in vivo experiments with bound peptides with *Staph*: **In progress**

Subtask 5B: Perform & analyze in vivo experiments with bound peptides with *A. baumanii*: **In progress**

Subtask 5C: Prepare & revise report(s)/manuscript(s) with results from Task 5: **To be performed**

4. **KEY RESEARCH ACCOMPLISHMENTS:**

* Developed murine model of orthopaedic implant infection.

* Documented that both *S. aureus* and *A. baumanii* impair implant osseointegration by inducing local osteolysis.

*Found that quorum sensing is required for *A. baumanii* infection to impair osseointegration.

*Found that IDR-1018 reduces the effects of *S. aureus* on implant osseointegration.

5. **CONCLUSION:** The murine model of orthopaedic implant infection will lead to future studies utilizing transgenic and knock out mice to understand the mechanisms that contribute to the effects of bacteria on implant osseointegration. The finding that *A. baumanii* impairs implant osseointegration by inducing local osteolysis reconciles confusion in the literature regarding the effects of *A. baumanii*. The finding that quorum sensing is required for *A. baumanii* infection to impair osseointegration will lead to future studies to determine whether quorum sensing antagonists that are being developed by other investigators will block the effects of *A. baumanii* on osseointegration. The finding that IDR-1018 reduces the effects of *S. aureus* on implant osseointegration will lead to future studies to optimize the use of IDR peptides in implant infection in our murine model; in larger, more clinically relevant, animal models; and ultimately in human trials.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. Manuscripts

      1. **Lay Press:** Nothing to report

      2. **Peer-Reviewed Scientific Journals:**

Choe H, Narayanan AS, Gandhi D, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM: IDR-1018: Immunomodulatory Peptide IDR-1018 Decreases Implant Infection and Preserves Osseointegration, Clin Orthop Rel Res, epub ahead of print, PMID:25953690

Choe H, Tatro JM, Corn DJ, Rettew A, Hausman B, Haku S, Gandhi D, Marshall S, Akkus O, Lee, Z, Bonomo R, Greenfield E: *Acinetobacter* and *Staphylococcus aureus* Impair Osseointegration of Orthopaedic Implants by

Macrophage-dependent Mechanisms, in preparation.

3. **Invited Articles:** Nothing to report

4. **Abstracts:**

Choe H, Tatro J, Corn D, Marshall S, Bosingnore L, Lee Z, Bonomo R, Greenfield E: Murine Model of Impaired Osseointegration due to Implant Infection. Military Health System Research Symposium, 2012.

Choe H, Tatro J, Corn D, Marshall S, Bosingnore L, Lee Z, Bonoma R, Greenfield E: Murine Model of Impaired Osseointegration due to Implant Infection. 57th Annual Meeting of the Orthopaedic Research Society, 2013.

Choe H, Corn D, Rettew A, Tatro J, Marshall S, Weinberg A, Lee Z, Bonoma R, Greenfield E: IDR-1018: A Synthetic Host Defense Peptide that Decreases Infection of Orthopaedic Implants. Military Health System Research Symposium, 2013.

Choe H, Corn D, Rettew A, Tatro J, Marshall S, Weinberg A, Wilber J, Marcus R, Lee Z, Bonoma R, Greenfield E: IDR-1018: A Synthetic Host Defense Peptide that Decreases Infection of Orthopaedic Implants. Annual Meeting of the American Society for Bone and Mineral Research, 2013.

Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonoma R, Greenfield E: *Acinetobacter* Impairs Osseointegration of Orthopaedic Implants in Mice. 58th Annual Meeting of the Orthopaedic Research Society, 2014.

Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonomo R, Greenfield E: Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice. AAOS/ORS Reseach Symposiuim on Musculoskeletal Infection. 2014.

Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonomo R, Greenfield E: Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice. 5th International Conference on Osteoimmunolgy. 2014.

Choe H, Narayanan AS, Corn DJ, Hausman BS, Haku S, Essber HA, Marshall SH, Lee Z, Bonomo RA, Greenfield EM: IDR-1018: An Immunodulatory Host Defense Peptide that Decreases Bacterial Burden and Preserves Osseointegration in a Murine Model of Orthopaedic Implant Infection, 59th Annual Meeting of the Orthopaedic Research Society, 2015.

Choe H, Bonomo RA, Rather PN, Greenfield EM: Mechanisms By Which *Staphylococcus* And *Acinetobacter* Inhibit Osseointegration, eCM XVI, 2015

b. **Presentations during the last year**

Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonoma R, Greenfield E: Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice. AAOS/ORS Reseach Symposiuim on Musculoskeletal Infection. 2014.

Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonoma R, Greenfield E: Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice. 5th International Conference on Osteoimmunolgy. 2014.

Choe H, Narayanan AS, Corn DJ, Hausman BS, Haku S, Essber HA, Marshall SH, Lee Z, Bonomo RA, Greenfield EM: IDR-1018: An Immunodulatory Host Defense Peptide that Decreases Bacterial Burden and Preserves Osseointegration in a Murine Model of Orthopaedic Implant Infection, 59th Annual Meeting of the Orthopaedic Research Society, 2015.

Choe H, Bonomo RA, Rather PN, Greenfield EM: Keynote Presentation: Mechanisms By Which *Staphylococcus* And *Acinetobacter* Inhibit Osseointegration, eCM XVI, 2015

7. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report

8. **REPORTABLE OUTCOMES:** Outcomes of our studies include the development of a murine model of orthopaedic implant infection; the finding that both *S. aureus* and *A. baumanii* impair implant osseointegration by inducing local osteolysis; the finding that quorum sensing is required for *A. baumanii* infection to impair osseointegration; and the finding that IDR-1018 reduces the effects of *S. aureus* on implant osseointegration.

9. **OTHER ACHIEVEMENTS:** Nothing to report

10. **REFERENCES:** Not applicable

11. **APPENDICES:**

Copy of the following publication:

Choe H, Narayanan AS, Gandhi D, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM: IDR-1018: Immunomodulatory Peptide IDR-1018 Decreases Implant Infection and Preserves Osseointegration, Clin Orthop Rel Res, epub ahead of print, PMID:25953690

Copies of the following posters:

#1: Choe H, Tatro J, Corn D, Marshall S, Bosingnore L, Lee Z, Bonomo R, Greenfield E: Murine Model of Impaired Osseointegration due to Implant Infection. 57th Annual Meeting of the Orthopaedic Research Society, 2013.

#2: Choe H, Corn D, Rettew A, Tatro J, Marshall S, Weinberg A, Wilber J, Marcus R, Lee Z, Bonoma R, Greenfield E: IDR-1018: A Synthetic Host Defense Peptide that Decreases Infection of Orthopaedic Implants. Annual Meeting of the American Society for Bone and Mineral Research, 2013.

#3: Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonoma R, Greenfield E: *Acinetobacter* Impairs Osseointegration of Orthopaedic Implants in Mice. 58th Annual Meeting of the Orthopaedic Research Society, 2014.

#4: Choe H, Rettew A, Hausman B, Haku S, Essber H, Marshall S, Akkus O, Rather P, Bonomo R, Greenfield E: Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice. AAOS/ORS Reseach Symposiuim on Musculoskeletal Infection. 2014.

Clin Orthop Relat Res
DOI 10.1007/s11999-015-4301-2

Clinical Orthopaedics and Related Research®
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SYMPOSIUM: RESEARCH ADVANCES AFTER A DECADE OF WAR

Immunomodulatory Peptide IDR-1018 Decreases Implant Infection and Preserves Osseointegration

Hyonmin Choe MD, PhD, Arvind S. Narayanan MS, Deep A. Gandhi, Aaron Weinberg DMD, PhD, Randall E. Marcus MD, Zhenghong Lee PhD, Robert A. Bonomo MD, Edward M. Greenfield PhD

Abstract

Background Innate defense regulator peptide-1018 (IDR-1018) is a 12-amino acid, synthetic, immunomodulatory host defense peptide that can reduce soft tissue infections and is less likely to induce bacterial resistance than conventional antibiotics. However, IDRs have not been tested on orthopaedic infections and the immunomodulatory effects of IDR-1018 have only been characterized in response to lipopolysacharide, which is exclusively produced by Gram-negative bacteria.

Questions/purposes We sought (1) to more fully characterize the immunomodulatory effects of IDR-1018, especially in response to *Staphylococcus aureus*; and (2) to determine whether IDR-1018 decreases *S aureus* infection of orthopaedic implants in mice and thereby protects the implants from failure to osseointegrate.

Methods In vitro effects of IDR-1018 on *S aureus* were assessed by determining minimum inhibitory concentrations in bacterial broth without and with supplementation of physiologic ion levels. In vitro effects of IDR-1018 on macrophages were determined by measuring production of monocyte chemoattractant protein-1 (MCP-1) and proinflammatory cytokines by enzyme-linked immunosorbent assay. In vivo effects of IDR-1018 were determined in a murine model of *S aureus* implant infection by quantitating bacterial burden, macrophage recruitment, MCP-1, proinflammatory cytokines, and osseointegration in nine mice per group on Day 1 postimplantation and 20 mice per group on Day 15 postimplantation.

Results IDR-1018 demonstrated antimicrobial activity by directly killing *S aureus* even in the presence of physiologic ion levels, increasing recruitment of macrophages to the site of infections by 40% ($p = 0.036$) and accelerating *S aureus* clearance in vivo ($p = 0.008$) with a 2.6-fold decrease in bacterial bioburden on Day 7 postimplantation.

One or more of the authors has received research funding from the Department of Defense Peer Reviewed Orthopaedic Research Program Idea Development Award (EMG), the Harry E. Figgie III MD Professorship (EMG), the Mochida Memorial Foundation for Medical and Pharmaceutical Research (HC), the Cleveland Department of Veterans Affairs (RAB), the Veterans Affairs Merit Review Program award number 1I01BX001974 (RAB), the Geriatric Research Education and Clinical Center VISN 10 (RAB), the National Institute of Allergy and Infectious Diseases of the National Institutes of Health awards R01AI063517 and R01AI10056 (RAB), and the Antibiotic Resistance Leadership Group under National Institutes of Health award UM1AI104681 (RAB).
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All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research*® editors and board members are on file with the publication and can be viewed on request.
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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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In vitro immunomodulatory activity of IDR-1018 included inducing production of MCP-1 in the absence of other inflammatory stimuli and to potently blunt excess production of proinflammatory cytokines and MCP-1 induced by lipopolysaccharide. Higher concentrations of IDR-1018 were required to blunt production of proinflammatory cytokines and MCP-1 in the presence *S aureus*. The largest in vivo immunomodulatory effect of IDR-1018 was to reduce tumor necrosis factor-α levels induced by *S aureus* by 60% ($p = 0.006$). Most importantly, IDR-1018 reduced *S aureus*-induced failures of osseointegration by threefold ($p = 0.022$) and increased osseointegration as measured by ultimate force (5.4-fold, $p = 0.033$) and average stiffness (4.3-fold, $p = 0.049$).

Conclusions IDR-1018 is potentially useful to reduce orthopaedic infections by directly killing bacteria and by recruiting macrophages to the infection site.

Clinical Relevance These findings make IDR-1018 an attractive candidate to explore in larger animal models to ascertain whether its effects in our in vitro and mouse experiments can be replicated in more clinically relevant settings.

Introduction

Orthopaedic implant infections are difficult to manage and often result in repeated surgical interventions and long-term treatment with intravenous antibiotics [24]. More than 100,000 orthopaedic implant infections occur in the United States each year and more than 80% are caused by *Staphylococcus aureus* [13]. Treatment of these infections is complicated by the fact that the surfaces of the orthopaedic plates, screws, and intramedullary nails used for fixation of the fractures provide ideal surfaces for formation of bacterial biofilms [18]. Moreover, prolonged infection and progressive inflammation result in osteolysis around the orthopaedic implants and, therefore, impair implant osseointegration and contribute to loss of fracture fixation [27]. Antibiotic resistance and the relative lack of new therapies in development further complicate treatment of orthopaedic implant infections, which are often associated with implant loosening, loss of fracture fixation, and fracture nonunion [31, 33, 41]. Additionally, increased incidence of methicillin-resistant *S aureus* infections is a major concern in both civilian and military orthopaedics [9, 29, 31, 45], because therapies for methicillin-resistant *S aureus* infections are limited.

Host-defense peptides (HDPs) represent a promising new approach to combating infection [6, 14, 15, 19]. HDPs were originally identified as small antimicrobial peptides produced by a wide variety of mammalian cell types. More recently, evidence has accumulated that the antiinfective effects of HDPs are also the result of their immunomodulatory properties such as upregulation of expression of chemokines that, in turn, recruit and activate host immune cells [6, 14, 15, 19, 28, 38]. HDPs can also modulate production of pro– and antiinflammatory cytokines to control the inflammatory response [14, 15, 19, 28, 38] and can inhibit osteoclast differentiation [20, 40] and thereby potentially can reduce inflammatory osteolysis and the lethal consequences of septic shock. As a result of their unique immunomodulatory mechanisms of action, HDPs are also less likely to induce bacterial resistance than are conventional antibiotics [14, 15]. Innate defense regulator peptide-1018 (IDR-1018) is a synthetic 12-amino acid derivative of bactenecin, a bovine HDP [42]. IDR-1018 can directly kill bacteria [42], can modulate differentiation and activation of macrophages and neutrophils, thereby regulating their production of chemokines and cytokines [1, 26, 30, 32, 37, 42], can reduce soft tissue infections [1, 37], and can disrupt bacterial biofilms [25, 35]. IDR-1018 was chosen as the focus of this study because it is the most potent IDR that has been described [37] and is therefore an attractive agent to potentially reduce orthopaedic infections and inflammatory osteolysis around orthopaedic implants. However, orthopaedic infections can be especially difficult to combat [18, 24] and IDRs have not been tested in that setting. Moreover, the immunomodulatory effects of IDR-1018 have only been characterized in response to lipopolysaccharide [32, 42], which is exclusively produced by Gram-negative bacteria.

The purposes of our study were therefore (1) to more fully characterize the immunomodulatory effects of IDR-1018, especially in response to *S aureus;* and (2) to determine whether IDR-1018 decreases *S aureus* infection of orthopaedic implants in mice and thereby protects the implants from failure to osseointegrate.

Material and Methods

IDR-1018 (VRLIVAVRIWRR-NH2) was synthesized, purified to 98.4% by high-performance liquid chromatography, and validated by mass spectrometry (Biomatik, Cambridge, Canada). IDR-1018 was dissolved in sterile water (10 mg/mL) and stored at −80° C.

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IDR-1018 has been reported to potently kill *S aureus* in bacterial broth [42]. However, physiologic ion levels block bactericidal effects of many HDPs [6, 19, 43]. We therefore determined the effect of physiologic ion levels on IDR-1018 minimum inhibitory concentrations using a modified broth microdilution method as recommended [11, 42, 43]. Thus, overnight cultures of bioluminescent, methicillin-sensitive *S aureus*-Xen36 (Caliper Life Sciences, Hopkinton, MA, USA) were diluted 100-fold in Mueller-Hinton broth (MHB; Difco, Franklin Lakes, NJ, USA) and incubated at 37° C until early log phase ($A_{600/0.1\ cm\ light\ path} = 0.05$) was reached (Nanodrop 1000; Fisher Scientific, Fair Lawn, NJ, USA). Bacterial suspensions were diluted to 5×10^5 colony-forming units (CFUs)/mL in MHB without or with physiologic ion supplementation (Table 1) and cultured overnight at 37 °C with 1/10 volume of 0.01% acetic acid containing 0.2% bovine serum albumin plus various concentrations of IDR-1018. Aliquots of the cultures were spotted on MHB agar plates, incubated overnight at 37° C, and the qualitative presence or absence of bacterial growth assessed visually as recommended [11, 42, 43].

To characterize the immunomodulatory effects of IDR-1018, production of cytokines and monocyte chemoattractant protein-1 (MCP-1) by RAW264.7 murine macrophages (ATCC, Manassas, VA, USA) was measured. RAW264.7 macrophages were maintained in minimum essential medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (Hyclone), nonessential amino acids (Cellgro, Manassas, VA, USA), L-glutamine (Cellgro), and penicillin/streptomycin (Cellgro) at 37 °C in 5% CO_2. RAW264.7 macrophages (1×10^5 cells in 2 mL of media/2-cm^2 well) were incubated with IDR-1018 (7−700 μg/mL) for 45 minutes and then incubated for 24 hours in the absence or presence of 2×10^7 CFU/mL heat-killed *S aureus* or ultrapure lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (InvivoGen, San Diego, CA, USA). Culture media were centrifuged (9000 g, 15 minutes) and supernatants stored at −20° C. MCP-1, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β were quantified by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Murine Model of Implant Infection

Our study was approved by our Institutional Animal Care and Use Committee. In vivo experiments focused on 1 day or 15 days after implantation (Fig. 1), respectively, because higher levels of cytokines, chemokines, and macrophage recruitment occur at the early time point and because preliminary studies showed that *S aureus* infection blocks osseointegration at 15 days. Female mice matched for age and strain were randomized to receive saline or IDR-1018 (200 μL, diluted to 1 mg/mL in saline; Henry Schein, Melville, NY, USA) intraperitoneally 4 hours before and 24 and 48 hours after implant insertion (Fig. 1). Each group contained nine mice in the 1-day experiments and 20 mice in the 15-day experiments. Titanium alloy screw-shaped implants (0.8 mm diameter, 3.5 mm length; Ti-6Al-4 V; Antrin Miniature Specialties Inc, Fallbrook, CA, USA) were rigorously cleaned with five cycles of alternating treatments in alkali ethanol (0.1 N NaOH and 95% ethanol at 32 °C) and 25% nitric acid [5]. Overnight cultures of bioluminescent *S aureus*-Xen36 were diluted 100-fold in lysogeny broth (LB; Fisher Scientific) and incubated at 37° C until early log phase was reached. Bacterial suspensions were centrifuged (1500 g, 5 minutes) and resuspended in 1/30 volume of LB (3×10^{10} CFUs/mL). Rigorously cleaned implants were incubated with 100 μL of the bacterial suspensions for 20 minutes at room temperature with gentle shaking, rinsed three times in phosphate-buffered saline (PBS), and immediately implanted into pilot holes in the middiaphysis of the femur [4, 5] of 6- to 8-week-old female C57BL/6 J or macrophage Fas-induced apoptosis (MAFIA) mice. Macrophage recruitment was measured in the MAFIA mice, in which the monocyte/macrophage-specific colony-stimulating factor receptor promoter drives both expression of enhanced green fluorescent protein and a Fas apoptosis system activated by the dimerization drug, AP20187 [8, 10]. MAFIA mice are on the C57BL/6 J background and have a normal phenotype unless exposed to AP20187. Macrophage recruitment was therefore quantified by fluorescence imaging with the Maestro imaging system (Perkin Elmer, Waltham, MA, USA) 1 day postimplantation after exposing the femurs, implants, and surrounding soft tissues by dissection. Fluorescence

Table 1. Bactericidal effects of IDR-1018

Bacterial culture media	Ca^{++} (mg/L)	Mg^{++} (mg/L)	NaCl (%)	MIC (μg/mL), mean ± SD
MHB	3.7	3.5	-	20 ± 13.9
MHB + physiological ion supplementation	28.7	16.0	0.9	38 ± 27.1
				p = 0.37 (t-test)

MIC = minimum inhibitory concentration; IDR-1018 = innate defense regulator peptide-1018; MHB = Mueller-Hinton broth; MICs for *Staphylococcus aureus*-Xen36 were determined in MHB media without or with physiological ion supplementation to the indicated final concentrations. MIC results represent mean ± SD of three independent experiments, each performed in duplicate

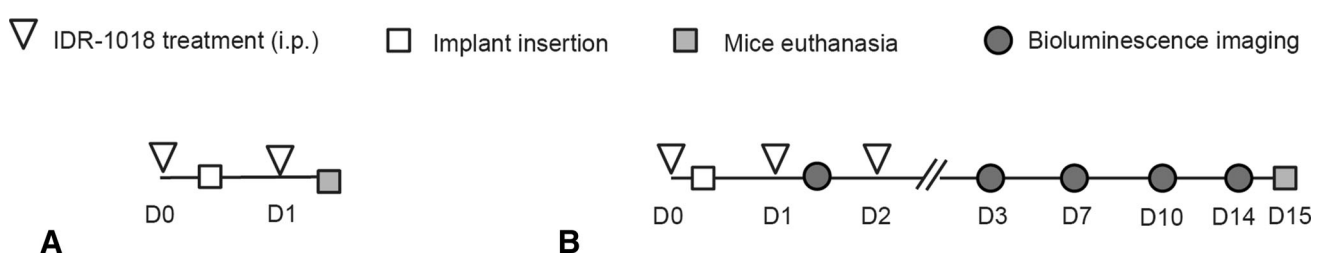

Fig. 1A–B Diagrams depict time lines for in vivo experiments. Mice were euthanized on (**A**) Day 1 or (**B**) Day 15 postimplantation. Mice received either saline or IDR-1018 (200 μg in 200 μL) intraperitoneally 4 hours before and 24 and 48 hours after implant insertion. Bioluminescence imaging was performed 1, 3, 7, 10, and 14 days postimplantation.

imaging signals from green fluorescent protein were defined by automatic spectral segmentation and were quantified in automatically selected regions of interest (ROIs) encompassing both the femurs and surrounding soft tissues.

Osseointegration failures were defined as implants that were not fixed in the femurs at the time of euthanasia and therefore could not be assessed by biomechanical pullout testing. Those implants were assigned values of 0 for the biomechanical measurements. Biomechanic pullout testing was performed immediately after euthanasia at a displacement rate of 1 mm/min as described previously [5]. Ultimate force, average stiffness, and work to maximum load were determined from the resultant load displacement curves according to ASTM standards (F543-07). To reduce preloading variability, calculations of stiffness and work began when ultimate force equaled 0.1 N.

S aureus-Xen36 contains a stable copy of the bacterial *lux ABCDE* operon at a single integration site and therefore emits bioluminescent signal without addition of exogenous substrate as long as the bacteria are viable [34]. Bioluminescence imaging was performed longitudinally on live mice using a Xenogen IVIS 200 system (Perkin Elmer/Caliper Life Sciences, Hopkinton, MA, USA) and data were analyzed using Xenogen Living Image 2.5 (Perkin Elmer/Caliper Life Sciences). Oval ROIs of the same size were placed on the femoral region encompassing the bioluminescence imaging signal for each mouse. Bioluminescence imaging signal intensity was quantified within each ROI on the indicated days postimplantation.

The numbers of CFUs and bacterial gene copies on the implants and in the surrounding femurs were quantified after pullout testing. Implants were sonicated for 10 minutes (50 W, 43,000 Hz) in 1 mL of PBS with 0.3% Tween-80 (Fisher Scientific, Fair Lawn, NJ, USA) followed by vortexing for 5 minutes [3]. Femurs were homogenized (Pro200H Series homogenizer; Pro Scientific, Oxford, CT, USA) in 500 μL of PBS and diluted with another 500 μL of PBS [3, 34]. CFUs in sonicates and homogenates were counted on LB agar plates. DNA was extracted from sonicates and homogenates (Power Biofilm™ DNA isolation kit; MoBio, Carlsbad, CA, USA). Real-time polymerase chain reaction assays with forward (5′–GACTTTCGCGTATT CGGCAC-3′) and reverse (5′-ATTGAGCAGCCCACT-CAGTC-3′) primers that target the *S aureus*-Xen36 *luxA* gene (Primer-BLAST, National Center for Biotechnology Information) were performed (Applied Biosystems 7500; Applied Biosystems, Foster City, CA, USA) using the standard curve method for quantification as we have done previously [12].

Femur homogenates were centrifuged (9000 g, 10 minutes) and supernatants stored at –20 °C. MCP-1, TNF-α, IL-6, and IL-1β were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

All error bars in the figures and tables show SDs. Statistical significance was determined by t-tests or by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests in experiments with multiple groups. Nonparametric Mann-Whitney tests, Kruskal-Wallis ANOVA followed by Newman-Kuels post hoc tests, or van Elteren's two-way tests were applied to data sets that were not normally distributed or were not of equal variance. Differences were reported as significant if the p value was < 0.05.

Results

Immunomodulatory Effects of IDR-1018

In vitro experiments demonstrated that, in the absence of inflammatory stimuli, high concentrations of IDR-1018 primarily act on macrophages to induce chemokine production and thereby recruit immune cells. On the other hand, in the presence of inflammatory stimuli such as occurs during bacterial infection, IDR-1018 primarily acted on macrophages to blunt excessive production of both proinflammatory cytokines and chemokines. Thus,

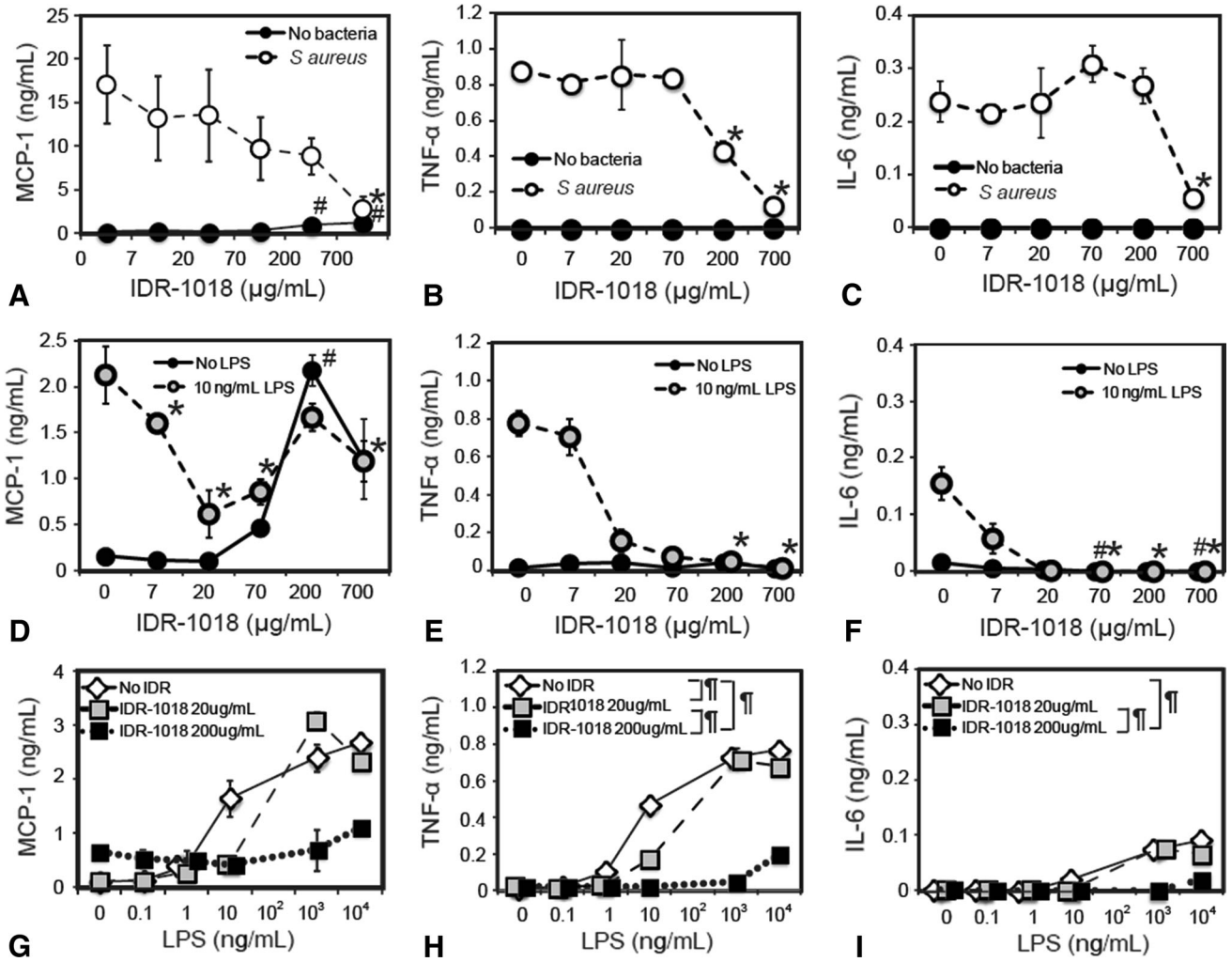

Fig. 2A–I IDR-1018 has in vitro immunomodulatory effects on macrophages. RAW264.7 macrophages were treated with the indicated concentrations of IDR-1018 45 minutes before stimulation with (**A–C**) 2×10^7 CFU/mL heat-killed *S aureus;* (**D–F**) ultrapure LPS at 10 ng/mL; or (**G–I**) ultrapure LPS at the indicated concentrations. Control wells received 40 μL of sterile water as vehicle controls for IDR-1018 and LPS. Cytokines and MCP-1 were measured by enzyme-linked immunosorbent assay in the culture media after 24-hour incubation. $^{\#}p < 0.05$ compared with control group without IDR-1018 and without other stimulus (**A–F**). $^{*}p < 0.05$ compared with control group without IDR-1018 but with either *S aureus* (**A–C**) or LPS (**D–F**). $^{¶}p < 0.05$ for overall effect of IDR-1018 (20 or 200 μg/mL; **G–I**). For data sets that were normally distributed and were of equal variance (white symbols in **A** and **C**, gray symbols in **D**, and black symbols in **A**), statistical analysis was by one-way ANOVA followed by Bonferroni versus control, post hoc tests. Otherwise, statistical analysis was by nonparametric Kruskal-Wallis ANOVA followed by Dunnett versus control, post hoc tests (white symbols in **B**, gray symbols in **E** and **F**, and black symbols in **B–F**) or by van Elteren's two-way tests (**G–I**). For **A**−**F**, n = 3 culture wells in each group; for **G–I**, n = 4 culture wells in each group. All results are representative of three to four experiments of each type and are reported as means ± SD.

relatively high concentrations (70–200 μg/mL) of IDR-1018 dose-dependently induced production of the chemokine MCP-1 (also known as CCL2 [black symbols in Figs. 2A, 2D]), but IDR-1018 did not induce production of TNF-α or IL-6 in the absence of other stimuli (Figs. 2B–C, 2E–F). Low concentrations (7–70 μg/mL) of IDR-1018 potently blunted LPS-induced production of MCP-1, TNF-α, and IL-6 (gray symbols in Fig. 2D–F) but higher concentrations (200–700 μg/mL) were required to blunt their production when induced by *S aureus* (open symbols in Fig. 2A–C). IL-1β levels in all of the groups were less than the lowest standard (3.9 pg/mL; data not shown), likely attributable to the absence of a signal 2 to activate inflammasome processing of pro–IL–1β to mature IL-1β [16]. LPS dose–response experiments showed that 20 μg/mL of IDR-1018 blunted the effects of low concentrations of LPS (10 ng/mL; gray symbols in Fig. 2G–I) but that 200 μg/mL of IDR-1018 was required to blunt the effects of a higher concentration of LPS (10^3–10^4 ng/mL; black symbols in Fig. 2G–I).

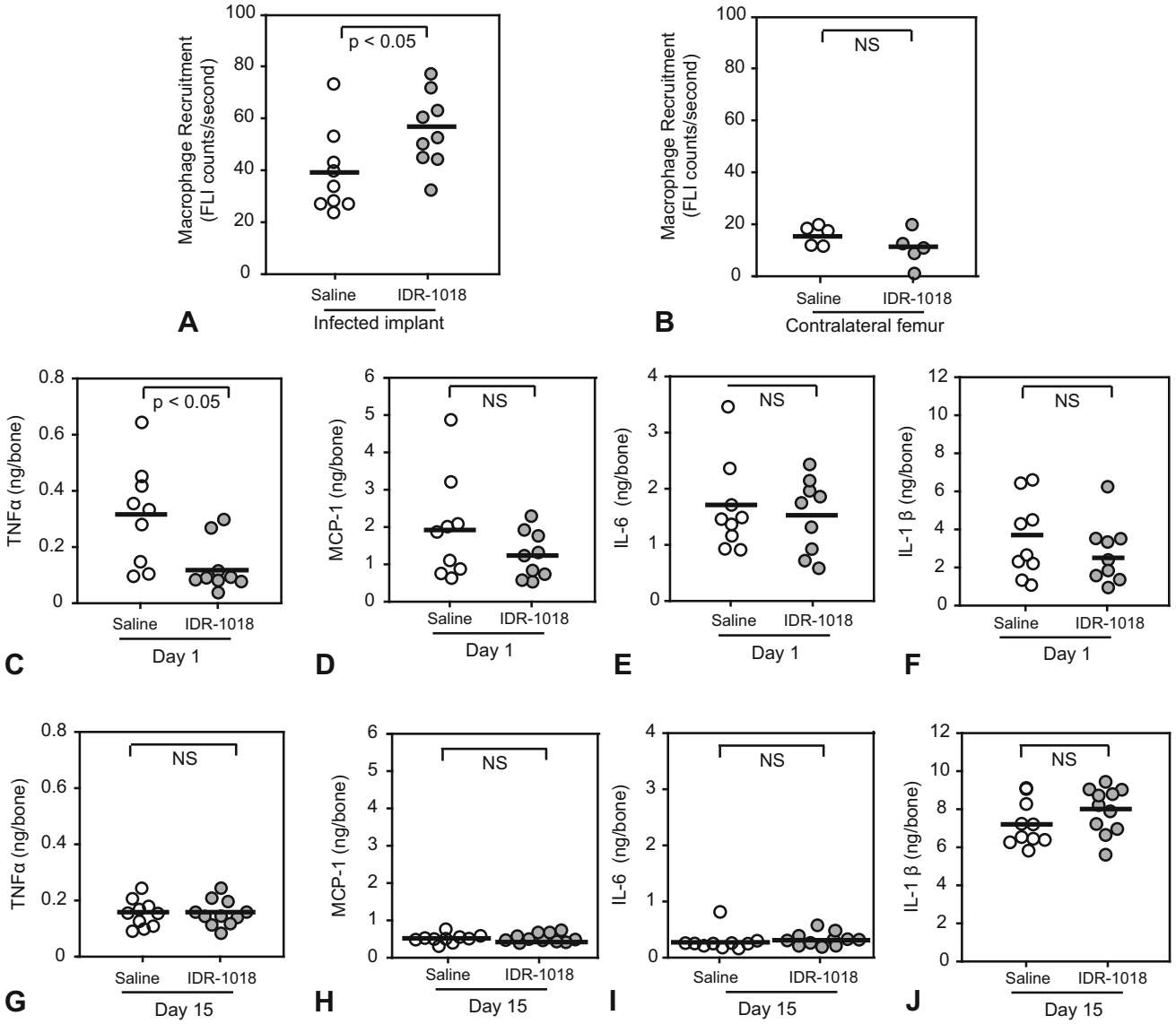

Fig. 3A–J IDR-1018 has in vivo immunomodulatory effects. IDR-1018 or saline was injected intraperitoneally 4 hours before and 24 and 48 hours after insertion of implants with adherent *S aureus*-Xen36. (**A–B**) Macrophage recruitment was measured by fluorescence imaging 1 day postimplantation. MCP-1, TNF-α, IL-6, and IL-1β were measured in the surrounding bones on (**C–F**) Day 1 or (**G–J**) Day 15 postimplantation. All data sets were normally distributed and were of equal variance; thus, black bars denote means and statistical analysis was performed using t-tests. For **A** and **C–J**, n = 9–11 mice per group; for B, n = 5 mice per group. NS = nonsignificant; FLI = fluorescence imaging.

The in vivo immunomodulatory effects of IDR-1018 were best observed at an early time point when higher levels of cytokines, chemokines, and macrophage recruitment occur (Fig. 1A). Thus, 1 day postimplantation, IDR-1018 enhanced recruitment of macrophages to the infected implant site by 40% ($p = 0.036$) without affecting macrophage levels at the contralateral femur (Fig. 3A–B). Consistent with the in vitro effects of IDR-1018 (Fig. 2), there was a 60% ($p = 0.006$) decrease in TNF-α level 1 day postimplantation (Fig. 3C), although there was no significant difference with the available number of samples in levels of MCP-1, IL-6, or IL-1β 1 day postimplantation (Fig. 3D–F). The levels of MCP-1 and the proinflammatory cytokines were relatively low on Day 15 and were unaffected by IDR-1018 (Fig. 3G–J).

Antibacterial Effects and Osseointegration

IDR-1018 killed *S aureus* in vitro and decreased *S aureus* infection of orthopaedic implants in mice. In vitro, IDR-1018 potently killed *S aureus* with similar minimum

inhibitory concentration values both in the absence and presence of physiologic ion levels (Table 1). One day postimplantation, IDR-1018 did not affect the in vivo bacterial burden (Fig. 4A–B). Consistent with those findings, longitudinal bioluminescence imaging demonstrated that IDR-1018 did not affect the initial bacterial burden but accelerated ($p = 0.008$) bacterial clearance between Days 1 and 7 postimplantation with a 2.6-fold difference in bioluminescence imaging on Day 7 (Fig. 4C). Bacterial clearance continued for a longer period of time in the absence of IDR-1018 such that the bacterial burdens were indistinguishable by Day 14 postimplantation (Fig. 4C–G).

IDR-1018 protected the implants from failure to osseointegrate. These experiments focused on 15 days after implantation (Fig. 1B) because preliminary studies showed that *S aureus* infection blocks osseointegration during that timeframe. IDR-1018 led to a threefold ($p = 0.022$) decrease in the frequency of the gross failures of osseointegration that are caused by *S aureus* infection in the murine model (Table 2). IDR-1018 also modestly increased biomechanic measures of osseointegration. Thus, ultimate force was increased by 5.4-fold ($p = 0.033$; Fig. 5A) and average stiffness was increased by 4.3-fold ($p = 0.049$; Fig. 5B), whereas work was not detectably affected (Fig. 5C). In contrast, gross osseointegration failures were never observed 1 day postimplantation and the biomechanic measures of osseointegration in the presence of *S aureus* infection were higher on Day 1 than on Day 15 (compare Fig. 5D–F with Fig. 5A–C).

Table 2. Gross integration failures at 15 days postimplantation

Outcome measure	*Staphylococcus aureus* implant infection	
	Saline	IDR-1018
Gross failures	12 mice	4 mice
Osseointegrated	8 mice	16 mice
Gross failure rate	60%	20%

$p = 0.022$ (Fisher's exact test); IDR-1018 = innate defense regulator peptide-1018

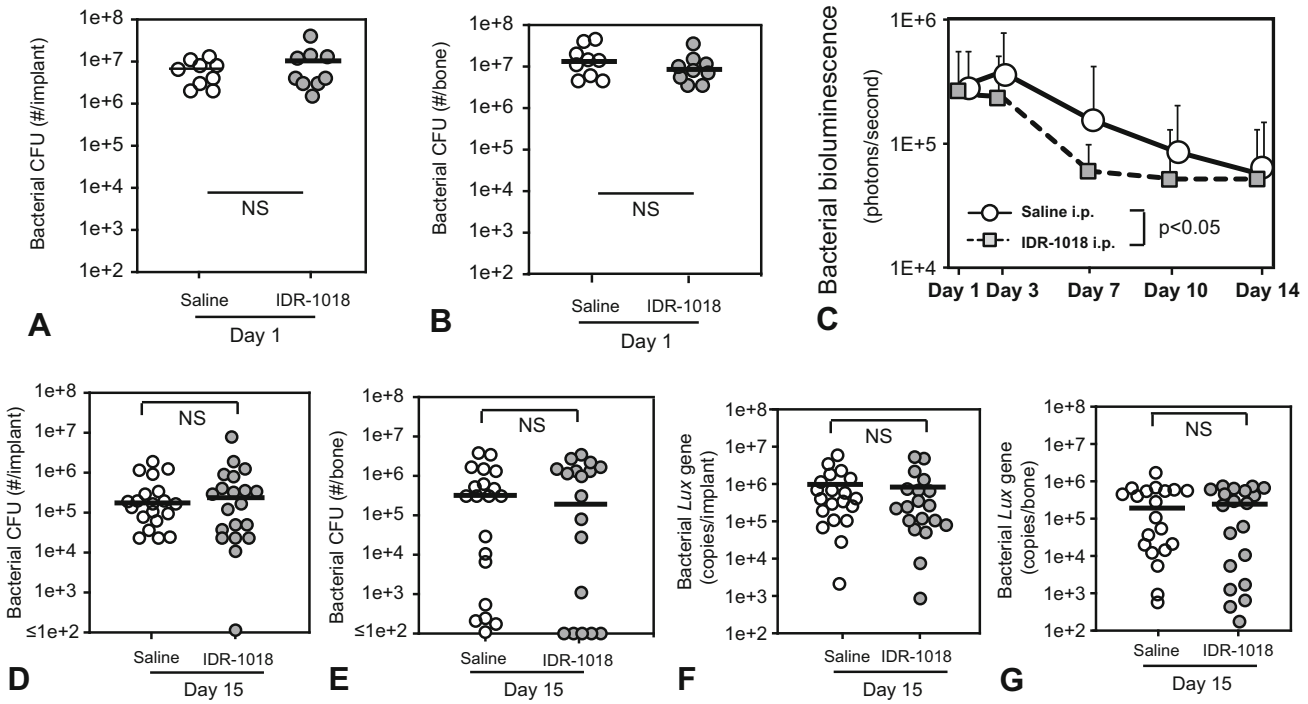

Fig. 4A–G IDR-1018 decreases implant infection. IDR-1018 or saline was injected intraperitoneally 4 hours before and 24 and 48 hours after insertion of implants with adherent *S aureus*-Xen36. Bacterial burden was determined on Day 1 postimplantation by measuring CFUs (**A**) adherent to the implants or (**B**) in the surrounding bones. Bacterial burden was determined (**C**) longitudinally by bioluminescence imaging. Bacterial burden was determined on Day 15 postimplantation by measuring (**D–E**) CFUs and (**F–G**) the *S aureus*-Xen36 *luxA* gene adherent to the implants (**D** and **F**) and in the surrounding bones (**E**, **G**). For data sets that were normally distributed and were of equal variance (**A–B**), black bars denote means with statistical analysis performed using t-tests. Otherwise, statistical analysis was by nonparametric, two-way van Elteren tests (**C**) or by nonparametric Mann-Whitney tests and black bars denote medians (**D–G**). For **A–B**, n = 9 mice per group; for **C–G**, n = 20 mice per group. NS = nonsignificant.

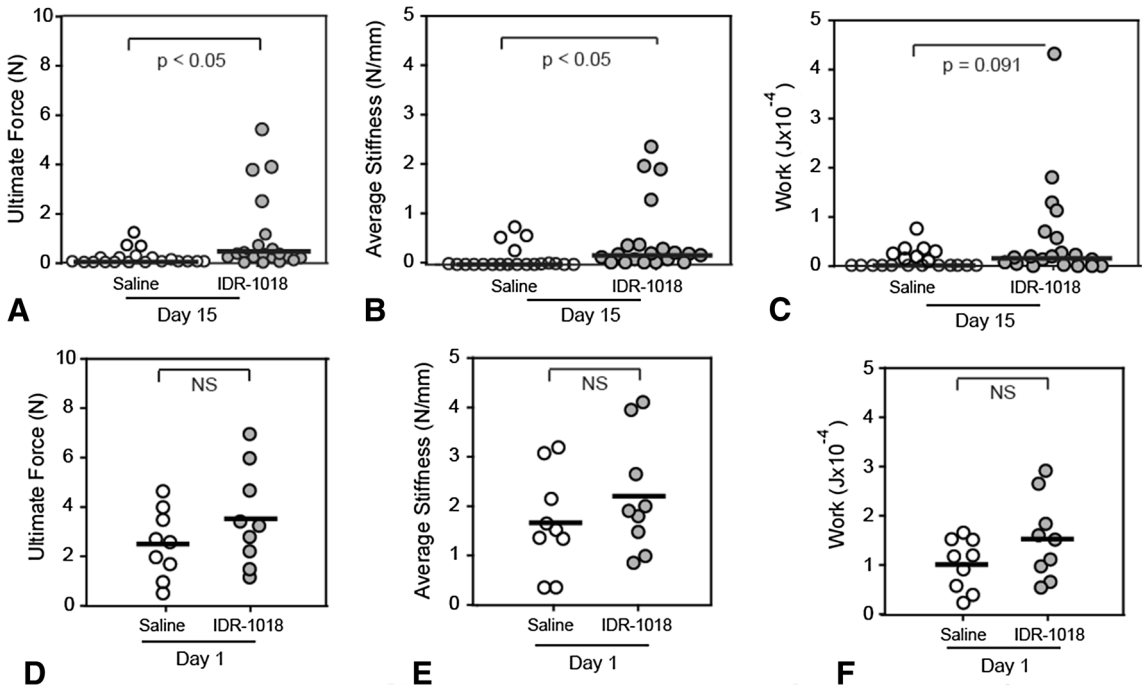

Fig. 5A–F IDR-1018 preserves osseointegration at 15 days after implant insertion. IDR-1018 or saline was injected intraperitoneally 4 hours before and 24 and 48 hours after insertion of implants with adherent *S aureus*-Xen36. Osseointegration was measured by biomechanic pullout testing on (**A–C**) Day 15 or (**D–F**) Day 1 postimplantation. For comparison, biomechanic measures of sterile implants at Day 15 after insertion were 11.6 ± 2.2 Newtons (N) for ultimate force, 18.5 ± 3.8 N/mm for average stiffness, and $6.1 \pm 1.6 \times 10^{-4}$ joules (J) for work to the maximum force point. For data sets that were normally distributed and were of equal variance (**D–F**), black bars denote means with statistical analysis performed using t-tests. Otherwise, statistical analysis was by nonparametric Mann-Whitney tests and black bars denote medians (**A–C**). For **A–C**, n = 20 mice per group; for **D–F**, n = 9 mice per group. NS = nonsignificant.

Discussion

Orthopaedic infections are difficult to manage because of the formation of bacterial biofilms on implant surfaces [18], the inflammatory osteolysis that causes osseointegration failures and loosening of infected implants [27], and antibiotic resistance [9, 23, 29, 31, 33, 41, 45]. HDPs are promising new agents [6, 14, 15, 19] that, owing to their unique immunomodulatory mechanisms of action, are less likely to induce bacterial resistance than are conventional antibiotics [14, 15]. Moreover, HDPs can also modulate cytokine production to control the inflammatory response [14, 15, 19, 28, 38] and thereby may reduce the risk of developing inflammatory osteolysis. IDR-1018 is one of the most attractive of the HDPs [1, 26, 30, 32, 37, 39, 42]. The purposes of our study were therefore (1) to more fully characterize the immunomodulatory effects of IDR-1018, especially in response to *S aureus;* and (2) to determine whether IDR-1018 decreases *S aureus* infection of orthopaedic implants in mice and thereby protects the implants from failure to osseointegrate.

A major limitation of our study is that IDR-1018 only partially reduced the bacterial burden, proinflammatory cytokine production, and osseointegration failures induced by *S aureus*. The partial effects might be explained by partial effects on free-living bacteria or by production of biofilms that protected the bacteria from the antimicrobial and immunologic effects of IDR-1018 [18]. In that regard, a limitation of our study is that the longitudinal bacterial bioluminesce data does not distinguish between free-living bacteria and bacteria within biofilms. Another limitation is that our study only assessed a single dose of IDR-1018, which was chosen based on its effectiveness in reducing soft tissue infections [1]. Nonetheless, IDR-1018 might be more effective if administered more often, at a higher concentration, or locally, at the infection site. IDR-1018 might also be more effective in combination with antibiotics and implant removal, which is the most likely clinical application. Another major limitation is that we only assessed effects of IDR-1018 on a single strain of *S aureus* and the results with other strains or other bacterial species could be different. We also only examined female mice, which were chosen because the previous study on IDR-

1018 and soft tissue *S aureus* infections was performed on female mice [1]. It is also always difficult to directly predict translational relevance in patients from results in rodents.

One primary purpose of our study was to more fully characterize the immunomodulatory effects of IDR-1018, especially in response to *S aureus*. We found that IDR-1018 was bactericidal even in the presence of physiologic ion levels, induced production of MCP-1, recruited macrophages to the site of infections, and blunted excess production of proinflammatory cytokines and MCP-1 induced by LPS in vitro or by in vivo infection with *S aureus*. However, the immunomodulatory effects of IDR-1018 were more complex than previously appreciated [1, 32, 42]. For example, our study was the first demonstration that IDR-1018 can blunt production of MCP-1. Moreover, IDR-1018 had relatively equivalent effects on production of cytokines and MCP-1 by macrophages in vitro, whereas in vivo, IDR-1018 had a greater effect on TNF-α levels, an intermediate effect on MCP-1, and little or no effect on IL-6. The difference between the in vitro and in vivo effects might be explained by macrophages being primarily responsible for TNF-α production in vivo [7], whereas IL-6 is primarily produced by other cell types [36] that are less affected by IDR-1018, and MCP-1 is produced by both macrophages and other cell types [44]. Consistent with this possibility, the relative in vitro cytokine levels in macrophage cell cultures compared with in vivo are highest for TNF-α, intermediate for MCP-1, and lowest for IL-6.

The other primary purpose of our study was to determine whether IDR-1018 decreases *S aureus* infection of orthopaedic implants in mice, thereby protecting the implants from failure to osseointegrate. Importantly, we found that IDR-1018 accelerated bacterial clearance and partially reduced both inflammatory osteolysis and infection-induced failure to osseointegrate. It is likely that decreased TNF-α levels, in response to treatment with IDR-1018, contributed to the improved osseointegration because the TNF-α levels were more strongly reduced than were the levels of other cytokines and chemokines that we measured. Moreover, TNF-α potently induces osteolysis [21] and contributes to osteolysis in other models of infection [2].

In conclusion, our results showed that IDR-1018 is potentially useful in reducing orthopaedic infections by directly killing bacteria, recruiting macrophages to the infection site, blunting excess cytokine production, and reducing osseointegration failures. IDR-1018 might therefore also reduce other examples of inflammatory osteolysis, including aseptic loosening of orthopaedic implants as a result of macrophage activation by wear particles [17, 22]. These findings make IDR-1018 an attractive candidate to explore in larger animal models to ascertain whether its effects in our in vitro and mouse experiments can be replicated in more clinically relevant settings. Additional studies to test and optimize possible strategies to increase the effectiveness of IDR-1018 are warranted. Future studies should also distinguish between effects of IDR-1018 on free-living bacteria and bacteria within biofilms and determine the effect of more potent IDRs as they are developed.

Acknowledgments We thank Ozan Akkus for advice on biomechanical testing; Nicholas M. Bernthal and Lloyd S. Miller for advice on murine implant infection models; David J. Corn for assistance with bioluminescence imaging and fluorescence imaging; Hani A. Essber, Sona Haku, and Jenifer Mikulan for assistance with the mouse surgeries; Susan W. Farmer and Robert E. W. Hancock for advice on handling IDR-1018; Bryan S. Hausman for assistance with both polymerase chain reaction primer design and mouse surgery; Steve H. Marshall for advice on microbiology methods; Eric Pearlman for providing the MAFIA transgenic mice; Mark D. Schluchter for statistical assistance; and Jack Wilber for suggestions on clinical relevance. We would like to acknowledge the support of the Case Center for Imaging Research which is a shared resource for Case Western Reserve University and the Case Comprehensive Cancer Center.

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Appended Poster #1

POSTER #: 1166

Murine Model of Impaired Osseointegration due to Implant Infection

Hyonmin Choe, David Corn, Joscelyn Tatro, Ashley Rettew, Steve Marshall, Lindsay Bonsignore, Zhenghong Lee, Robert Bonomo, Edward Greenfield

Case Western Reserve University and Louis Stokes Cleveland VA Medical Center

Introduction

Infection is the most difficult complication of orthopaedic implant surgery and often impairs osseointegration of the implant. Development of novel therapeutic agents is therefore required. Murine models are extremely useful for this purpose due to the availability of transgenic and knockout strains. Moreover, the reduced cost of mice compared to larger animals facilitates screening of novel therapeutic strategies. However, a murine infection model that assesses implant osseointegration has not yet been established.

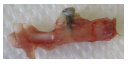
In this study, we compared sterile implants and implants with adherent bacteria based in our previously validated murine model of osseointegration [1,2].

Material and Methods

1.0 $\times 10^5$ (low dose) or 3×10^6 (high dose) CFUs / implant of bioluminescent *Staph. aureus* (Xen36 strain, Caliper Life Sciences) were adhered to rigorously cleaned titanium alloy screw shaped implants (1 mm diameter, 3.2 mm length) immediately before insertion into pilot holes in the mid-diaphysis of the femur of 7 week old male C57BL/6 mice [1-2].

We quantified the bacterial burden by bioluminescence imaging (BLI: Xenogen IVIS 200 system) and counting of colony forming units (CFUs), and *Lux* specific real-time PCR for sonicated Implants and homogenized femurs [3].

Osseointegration was measured by histomorphometry and biomechanical pull-out testing [1,2].

Dissected femur 

Results

• No bacteria group • Low dose group • High dose group

Fig. 1: Bioluminescence imaging (BLI)

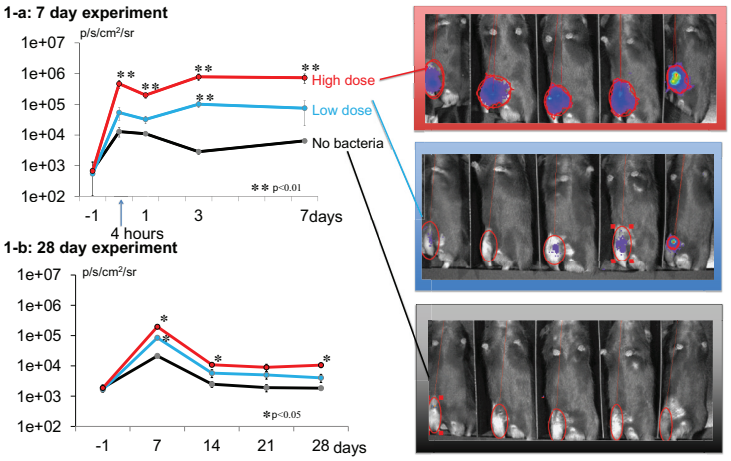


Fig. 2: CFUs at day7

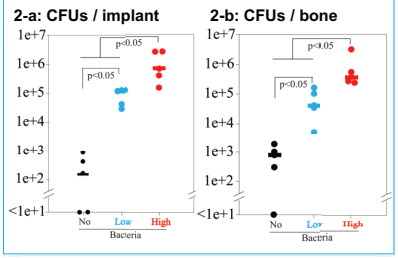


Fig. 3: *Lux* gene at day7

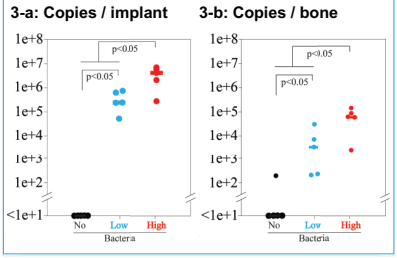
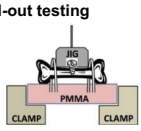


Fig. 4: Osseointegration

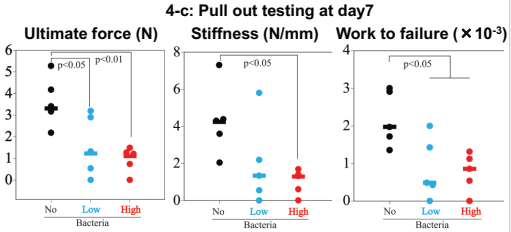
4-a: Gross integration failures

	No bacteria	Low dose	High dose
7 day	0/5	1/5	1/5
28 day	0/5	0/5	2/5

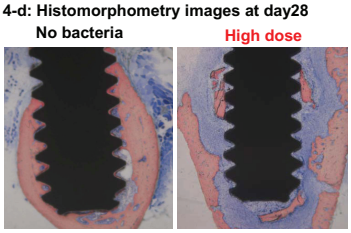
4-b: Pull-out testing



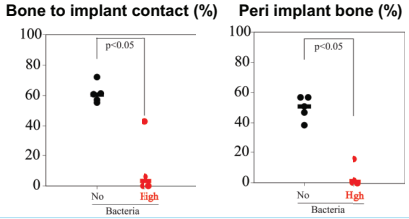
4-c: Pull out testing at day7



4-d: Histomorphometry images at day28



4-e: Histomorphometry at day28



Discussion

1. Signs of systemic sepsis were not observed in any of the mice and the BLI imaging demonstrated that the infection was localized to the implant site.
2. In mice without bacteria, BLI was low at all time points (Fig 1).
3. Bacterial inoculation increased BLI (Fig 1), CFUs, and amount of *Lux* gene in a dose-dependent fashion (Fig 2 and 3).
4. Osseointegration was significantly decreased by bacteria as assessed by the frequency of gross integration failures, pull-out testing, and histomorphometry. (Fig 4).
5. The long-term ability to detect BLI is likely due to the use of *S. aureus* with the *lux* operon in a stable plasmid as shown by other investigators [3].
6. With this model, we are now in a position to assess novel methods of preventing/treating infections.

Conclusion

We have developed a quantitative and reproducible murine model of orthopaedic implant infection that includes impaired osseointegration.

References

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[3] Bernthal et al, 2010 PLoS ONE 5:e12580

Acknowledgement

Dept of Defense
Peer Reviewed Orthopaedic Research Program
Idea Development Award

Appended Poster #2

Poster #MO0160

IDR-1018: A Synthetic Host Defense Peptide that Decreases Infection of Orthopaedic Implants

Hyonmin Choe, David J Corn, Ashley N Rettew, Joscelyn M Tatro, Steve H Marshall, Aaron Weinberg, Zhenghong Lee, Robert A Bonomo, Edward M Greenfield

Case Western Reserve University and Louis Stokes Cleveland VA Medical Center

Introduction

Orthopaedic implant infection is becoming increasingly difficult to treat due to the prevalence of multiple-drug-resistant bacteria. Host defense peptides are less likely than antibiotics to induce bacterial resistance because they decrease infection primarily by recruiting and activating macrophages rather than by directly killing the bacteria [1]. IDR-1018 is a novel small synthetic host defense peptide that decreases soft tissue infections [2 and 3].

The purpose of the current study was to determine whether IDR-1018 decreases implant infection and would therefore prevent impaired osseointegration due to infection.

Materials and Methods

Bacteriostatic and bactericidal effects on *Staphylococcus aureus*

The minimal inhibitory and minimal bactericidal concentration (MIC and MBC) of IDR-1018 on *S. aureus* were determined in both Mueller Hinton Broth and physiological saline.

Effects on implant infection:

- Bioluminescent S. *aureus* (Xen36 strain, Caliper Life Sciences) were adhered to titanium alloy implants (1 mm diameter, 3.2 mm length) before insertion into pilot holes in the mid-diaphysis of the femur of 6-8 week old mice [4 and 5].
- IDR-1018 (200ug/injection) or saline was administrated i.p. daily beginning 4 hours before implant insertion and 24 and 48 hours after implant insertion.
- Mice were euthanized at 15 days post operation.
- Bacterial burden was quantified by non-invasive bioluminescence imaging (BLI: Xenogen IVIS 200 system) as well as by counting colony forming units (CFUs) and *Lux* gene on the implants and in the bones.
- Osseointegration was measured by biomechanical pull-out testing as previously described for this murine implant model [4 and 5].

Results

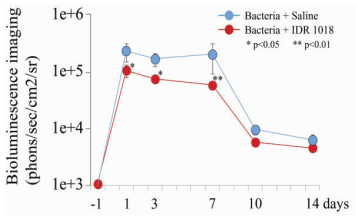
Fig 1. IDR-1018 Reduces Bacterial Burden

- Although IDR-1018 less directly reduces bacteria in a solution with physiological ionic strength than in a broth (A), it reduced the overall bacterial burden assessed by BLI at days 1, 3 and 7 after operation (B).
- IDR-1018 also reduced CFUs and *lux* gene in the bones at day 7 but had less effect on bacteria adherent to the implant (C and D) .

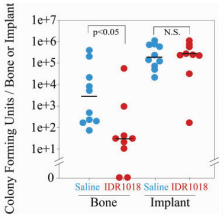
A. MIC and MBC on S. *aureus*

	MIC (μg/ml)	MBC (μg/ml)
Bacterial Broth	4	16
Saline	32	64

B. BLI



C. CFU



D. *Lux* gene

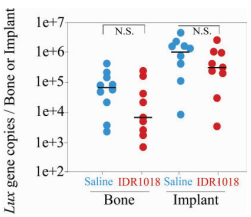


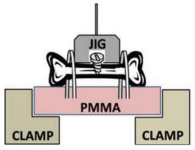
Fig 2. IDR-1018 Increases Osseointegration

A. Gross integration Failure rate

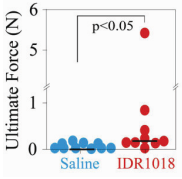
	(Failure / Total mice)
Saline	60% (6 / 10)
IDR1018	33% (3 / 9)

- IDR1018 reduced the gross integration failure rate and increased ultimate force and stiffness.

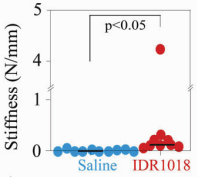
B. Pull out-testing



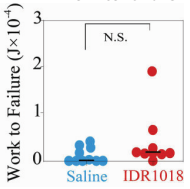
C. Ultimate Force



D. Stiffness



E. Work to failure



Discussion

IDR-1018

- reduced implant infection and therefore prevented impaired osseointegration (Fig 1 and 2).
- has less effect on the bacteria adherent to the implant because biofilm-formation on the implant likely protect the bacteria from the antibacterial effect of IDR1018.
- potentially acts by recruiting and/or activating inflammatory cells [3] rather than by directly killing the bacteria in physiological ionic strength (Fig1.A) and therefore is unlikely to induce bacterial resistance.

Future study

IDR-1018 may be more effective in combination with antibiotics and/or implant removal. Evaluation of these possibilities is especially important because IDR-1018 is likely to be clinically used in combination with both antibiotics and implant removal.

Acknowledgement

1. Dept of Defense, Peer Reviewed Orthopaedic Research Program Idea Development Award
2. Mochida Memorial Foundation for Medical and Pharmaceutical Research

References

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[2] Steinstraesser et al, 2013 PLOS ONE 8(1): e52449
[3] Wieczorek et al, 2010, Chemistry and Biology17: 970-980
[4] Bonsignore et al, 2011 Bone 49: 923-930
[5] Choe et al, 2013, ORS poster #1166

Appended Poster #3

2028

Acinetobacter Impairs Osseointegration of Orthopaedic Implants in Mice

Hyonmin Choe[1], Ashley N. Rettew[1], Bryan S. Hausman[1], Sona Haku[1], Hani A. Essber[1], Steve H. Marshall[2], Ozan Akkus[1], Phillip N. Rather[3, 4], Robert A. Bonomo[1, 2], and Edward M. Greenfield[1]

Case Western Reserve University[1], Louis Stokes Cleveland VA Medical Center[2], Emory University School of Medicine[3], and Atlanta Veterans Affairs Medical Center[4]

Introduction

1. Acinetobacter baumannii and closely related ***A. nosocomialis:***

- cause combat trauma-related osteomyelitis
- becoming prevalent in civilian osteomyelitis [1-3].
- reported to increase osteogenesis around non-osseointegrating stainless steel implants [4]

2. Quorum sensing (QS):

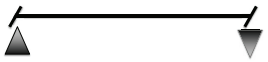
Bacterial cell-cell communication system that signals cell density and induces production of virulence factors and biofilms.

Goals are to determine whether:

- *Acinetobacter* affects osseointegration in our murine model of implant infection.
- QS or biofilm formation are responsible for the effects of *Acinetobacter* on osseointegration.

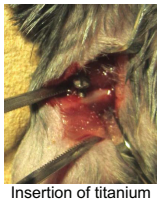
Material and Methods

Murine model of implant infection [5] **with M2 strain (*Acinetobacter nosocomialis*)** [3]



Day 0: Implantation **Day 7: Assessment**

BL6 mice (6-8wks, female)



Insertion of titanium alloy implants (1 mm diameter, 3.2 mm length)

Osseointegration:

- Histomorphometry
- Pull-out testing

Bacterial Bioburden:

- CFUs on implant and in femur

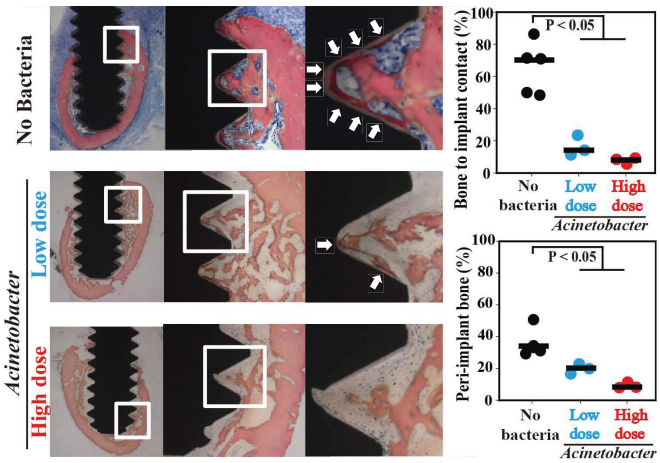
Pro-inflammatory Cytokines

- IL-1β, IL-6, and TNF-α,

1. **Effect of *Acinetobacter* on osseointegration:**
   - **No bacteria group**
   - ***Acinetobacter* group**
     Low dose; **$4x10^6$ CFUs/ implant**
     High dose; **$3x10^7$ CFUs/ implant**
2. **Effect of QS and biofilm on osseointegration:**
   - **Wild-type *Acinetobacter***
   - ***abal* gene deficient mutant**:
     No QS and reduced biofilm development [6].
   - **Bap gene deficient mutant**:
     Stronger reduction in biofilm formation, intact QS [7].

Results

Figure 1. Osseointegration (histomorphometry)



White arrows point the bone to implant contacts

Figure 2. Osseointegration (pull-out testing)

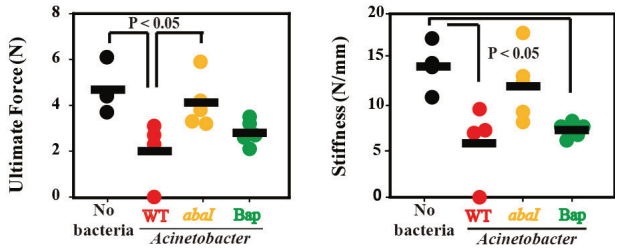


Figure 3. Bacterial Bioburden

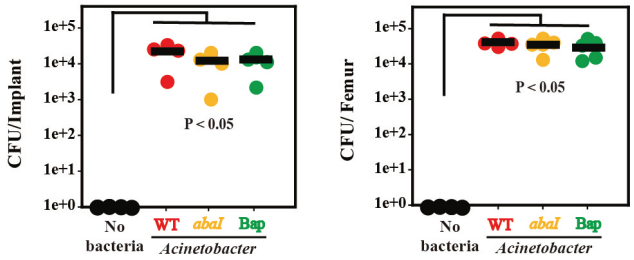
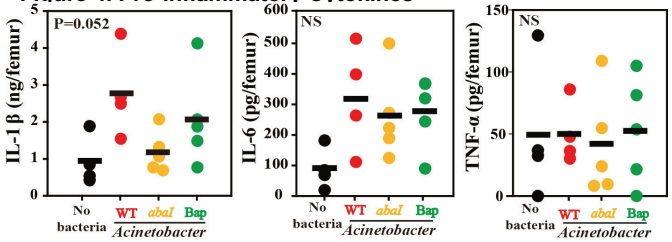


Figure 4. Pro-inflammatory Cytokines

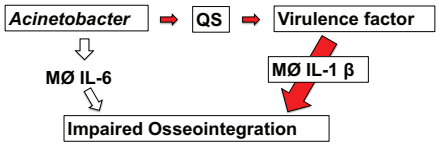


Discussion

1. *Acinetobacter* infection impairs osseointegration (Figure 1 and red symbols in Figure 2).
2. Osseointegration was not affected by the mutant strain deficient in QS (yellow symbols in Figure 2).
3. Mutant strain with reduced biofilm formation has similar effects on osseointegration as wild type *Acinetobacter* (green symbols in Figure 2).
4. Neither mutant altered the number of bacteria on the retrieved implants or in femurs (Figure 3).
5. QS specifically affects IL-1β production without altering IL-6 or TNF- α (Figure 4).
6. Novel therapeutics that disrupt QS may therefore be especially useful for treatment of implants infected with *Acinetobacter* or other QS-dependent bacteria.
7. Previous report [4] that *Acinetobacter* increases osteogenesis may be due to use of QS deficient strain or non-osseointegrating implants.

Conclusion

***Acinetobacter* QS dependent virulence factors cause impaired osseointegration.**



Future Directions

1. Increase *N.*
2. Identify virulence factors induced by QS that cause impaired osseointegrartion
3. Determine whether IL-1 β is responsible for *Acinetobacter*-induced osteolysis.

References

[1] Davis et al, 2005, Emerg Infect Dis, 11(8):1218-24.
[2] Nemec et al, 2011, Res Microbiol, 162(4): 393-404.
[3] Carruthers et al, 2013, Gen Announ 7; 1(6): e00906-13.
[4] Crane et al, 2009, J Orthop Res, 27: 1008-15.
[5] Choe et al, 2013, ORS#1166.
[6] Niu et al, 2008, J Bacteriol, 190(9): 3386-3392.
[7] Loehfelm et al, 2008, J Bacteriol, 190(3): 1036-44.

Acknowledgements

Department of Defense Peer Reviewed Orthopaedic Research Program Idea Development Award (EMG), Mochida Memorial Foundation for Medical and Pharmaceutical Research (HC), the NIH (PNR, RAB) and the VA Merit Award Program (PNR, RAB).

Appended Poster #4

Quorum Sensing is Required for *Acinetobacter* Infection to Impair Osseointegration of Orthopaedic Implants in Mice

Hyonmin Choe[1], Ashley N. Rettew[1], Bryan S. Hausman[1], Sona Haku[1], Hani A. Essber[1], Steve H. Marshall[2], Ozan Akkus[1], Phillip N. Rather[3, 4], Robert A. Bonomo[1, 2], and Edward M. Greenfield[1]

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Goals are to determine whether:

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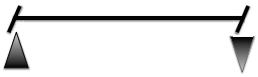
- QS or biofilm formation are responsible for the effects of *Acinetobacter* on osseointegration.

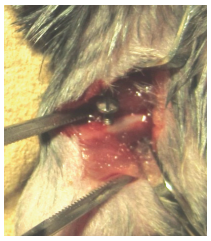
Material and Methods

Murine model of implant infection [5] **with M2 strain (*Acinetobacter nosocomialis*)** [3]



Day 0: Implantation **Day 7: Assessment**

BL6 mice (6-8wks, female)



Insertion of titanium alloy implants (1 mm diameter, 3.2 mm length)

Osseointegration:

- Histomorphometry
- Pull-out testing

Bacterial Bioburden:

- CFUs on implant and in femur

Pro-inflammatory Cytokines

- IL-1β, IL-6, and TNF-α,

1. Effect of *Acinetobacter* on osseointegration:

- **No bacteria group**
- ***Acinetobacter* group**
  - **Low dose; 4x10⁶ CFUs/ implant**
  - **High dose; 3x10⁷ CFUs/ implant**

2. Effect of QS and biofilm on osseointegration:

- **Wild-type *Acinetobacter***
- ***abaI* gene deficient mutant**:

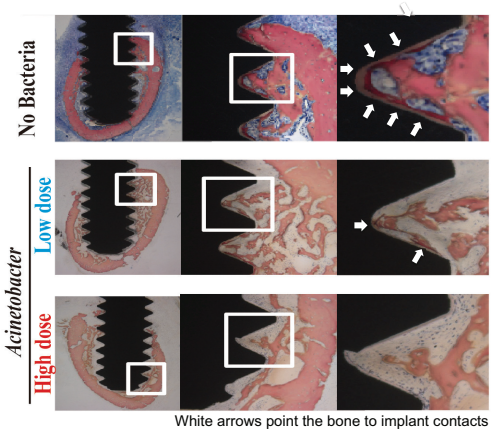
No QS and reduced biofilm development [6].

- **Bap gene deficient mutant**:

Stronger reduction in biofilm formation, intact QS [7].

Results

Figure 1. Osseointegration (histomorphometry)



White arrows point the bone to implant contacts

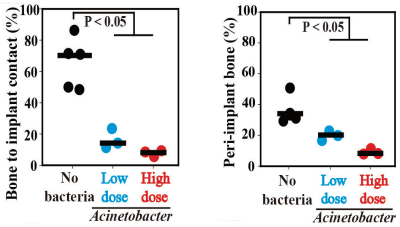


Figure 2. Osseointegration (pull-out testing)

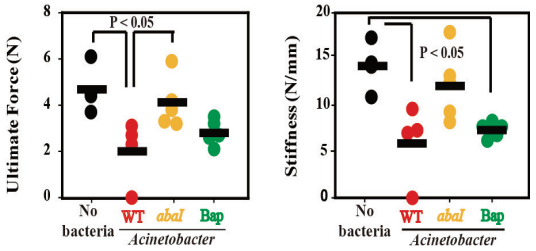


Figure 3. Bacterial Bioburden

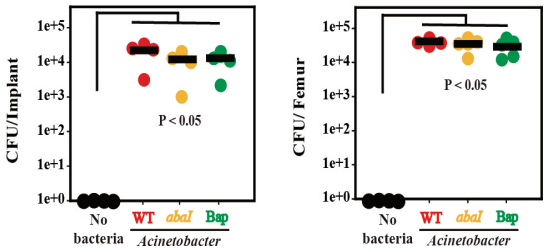
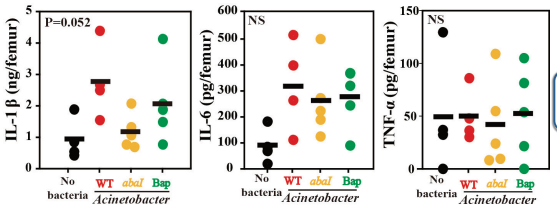


Figure 4. Pro-inflammatory Cytokines

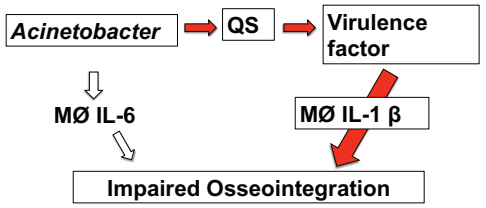


Discussion

1. *Acinetobacter* infection impairs osseointegration (Fig 1 and red symbols in Fig 2).
2. Osseointegration was not affected by the mutant strain deficient in QS (yellow symbols in Fig 2).
3. Mutant strain with reduced biofilm formation has similar effects on osseointegration as wild type *Acinetobacter* (green symbols in Fig 2).
4. Neither mutant altered the number of bacteria on the retrieved implants or in femurs (Fig 3).
5. QS specifically affects IL-1β production without altering IL-6 or TNF- α (Fig 4).
6. Novel therapeutics that disrupt QS may therefore be especially useful for treatment of implants infected with *Acinetobacter* or other QS-dependent bacteria.
7. Previous report [4] that *Acinetobacter* increases osteogenesis may be due to use of QS deficient strain or non-osseointegrating implants.

Conclusion

***Acinetobacter* QS dependent virulence factors cause impaired osseointegration.**



Future Directions

1. Increase *N.*
2. Identify virulence factors induced by QS that cause impaired osseointegrartion
3. Determine whether IL-1 β is responsible for *Acinetobacter*-induced osteolysis.

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Acknowledgements

Department of Defense Peer Reviewed Orthopaedic Research Program Idea Development Award (EMG), Mochida Memorial Foundation for Medical and Pharmaceutical Research (HC), the NIH (PNR, RAB) and the VA Merit Award Program (PNR, RAB).